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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Shin-Ichi Funahashi et al. Art Unit : 1646
Serial No. : 09/502,698 Examiner : P. Mertz
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Title : PROTEIN HAVING PDZ DOMAIN SEQUENCE

Commissioner for Patents
Washington, D.C. 20231

Considered by the Examiner PM 12/23/02

DECLARATION OF

SHIN-ICHI FUNAHASHI UNDER 37 CFR §1.132

1. I, Shin-Ichi Funahashi, having an address at 153-2, Nagai, Niihari-mura, Niihari-gun, Ibaraki, JAPAN, am a co-inventor of the above-captioned United States patent application serial No. 09/502,698.

2. My laboratory used a yeast two-hybrid assay employing PDZ domains from SEQ ID NO:1 and 2 as "bait". SEQ ID NO:1 contains PDZ domains designated in the specification as E-M, while SEQ ID NO:2 contains PDZ domains F-M. In the present assay, we utilized PDZ domains I-K and L-M (following the nomenclature of the specification). The yeast strain used was PJ69-4A and the method was in accordance with the protocol from Clontech. The screening was carried out by using an aorta cDNA library "Human Aorta MATCHMAKER cDNA library (HL4040AH)" and a prostate cDNA library "Human Prostate MATCHMAKER cDNA library (HL4037AH)." Most of the PDZ-binding proteins identified by using these domains I-K and L-M contained the consensus

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sequence Ser/Thr-Xaa-Val/Ile/Leu at their carboxy termini (Xaa represents an arbitrary amino acid).

Construction of vectors

PCR was carried out using, as a template, pBS-3372-6551 containing the region of nucleotides 336-6545 of SEQ ID NO:86. The composition of the reaction mixture was as follows: 5 μl of 10x KOD buffer #1 accompanying a TOYOBO KOD DNA polymerase (TOYOBO #KOD-101), 2 μl of 25 mM $MgCl_2$, 4 μl of 2.5 mM dNTPs, 1 μl of pBS-3372-6551 DNA, 35 μl of distilled water, 1 μl of KOD dash DNA polymerase and 1 μl of 10 μM primers. 50 μl of the prepared reaction mixture was placed in a 0.2-ml tube. The thermal cycling profile consisted of pre-heating at 94°C for 1 minute; 5 cycles of denaturation at 94°C for 15 seconds, annealing at 50°C for 30 seconds and extension at 74°C for 30 seconds; and 15 cycles of denaturation at 94°C for 15 seconds, annealing at 50°C for 30 seconds and extension at 74°C for 30 seconds.

The PCR products amplified in this reaction were fractionated by agarose electrophoresis. A gel piece containing the band with the desired PCR product was excised from the gel and the DNA contained in the excised gel was purified with a QIAquick Gel Extraction kit (QIAGEN #28706). The PCR product IK corresponds to nucleotides 4459-5501 of the 32-8-1 gene and was amplified by using primer 9N (5'-ATGCCAACTGTTACTACTTCTG-3') and primer 4C (5'-GACTCATGGACCAGCTTTGAT-3'). The PCR product LM corresponds to nucleotides 5573-6283 of the 32-8-1 gene and was amplified by using primer 5N (5'-ATGCTCTCTGGATCCAGTACAT-3') and primer 6C (5'-GACTCAAGAGAGAACCATCAAAG-3'). The ends of the PCR products were treated with T4 DNA polymerase. 30 μl of a reaction mixture was prepared containing 3.5 μl of 10x T4 DNA polymerase buffer accompanying TAKARA #2040A T4 DNA polymerase), 2 μl of the PCR products, 1.5 μl of 2 mM dGTP, 27 μl of sterilized water and 3 units of T4 DNA polymerase (TAKARA #2040A). After the mixture was incubated at 12°C for 30 minutes, the enzyme was inactivated at 80°C. 5 times as much volume of

Buffer PB (QIAGEN #28106 QIAquick PCR purification kit) was added to the mixture. The resulting mixture was agitated well. The DNA was eluted with 30 µl of sterilized water according to the manual.

A yeast expression vector pODB80 (Biotechniques Vol. 23, p.816; a gift from Dr. Olivier Louvet at Bordeaux University, France) used to clone the PCR product was pre-digested with restriction enzymes *NcoI* and *SalI*. 1 µg of the pre-digested vector was combined with 3 µl of 10x Klenow buffer (accompanying TAKARA #2140 Cloned Klenow fragment), 1 µl of 2 mM dCTP, 2 mM dTTP and 4 units of Klenow fragment (TAKARA #2140). The total volume of this mixture was adjusted to 30 µl and the mixture was incubated at room temperature for 15 minutes. Then the enzyme was inactivated by heating at 75°C for 15 minutes. 5 times as much volume of buffer PB (QIAGEN #28106 QIA quick PCR purification kit) was added to the mixture and the resulting solution was mixed well. The DNA was eluted with 30 µl of sterilized water according to the manual. The PCR products treated with T4 DNA polymerase and the vector treated with Klenow fragment, both of which have cohesive ends, were combined and ligated with each other. A TAKARA DNA Ligation Kit Ver.2 (TAKARA #6022) was used for the ligation. 10 µl of DNA ligation solution I was combined with 2 µl of the vector and 8 µl of the PCR products, and the mixture was incubated at 16°C for 30 minutes. *E. coli* was transformed with the ligate. The nucleotide sequence of the plasmid DNA, which had been extracted by a conventional method, was determined with a Big Dye™ Terminator Cycle Sequencing FS Ready Reaction Kit (ABI #4303151) in an automatic sequencer ABI PRISM 377XL. The resulting vectors, pODB80-PDZ IK and pODB80-PDZ LM, were used for yeast PJ69-4A transformation as described below.

Preparation of yeast transformants

Each of pODB80-PDZ IK and pODB80-PDZ LM was introduced into yeast PJ69-4A (Philip James, Genetics 144:1425-1436, 1996). The method used was as described in Gietz, R.D. and R.H. Schiestl, Methods in Molecular and Cellular Biology 5:255-269 (1995). The overnight culture of PJ69-4A was centrifuged at 2400 rpm for 5 minutes. The

precipitated yeast cells were suspended in 1ml of sterilized water and then transferred into a 1.5-ml tube. The yeast cells were precipitated again by centrifugation at 14000 rpm for 5 seconds. After the supernatant was removed, 900 µl of sterilized water and 100 µl of 1 M lithium acetate (pH 8.7) were added thereto and the mixture was stirred well with a vortex. The suspension was incubated at 30°C for 5 minutes. After centrifugation at 14000 rpm, the supernatant was removed, 240 µl of 50% polyethylene glycol 4000, 36 µl of 1M lithium acetate (pH 8.7), 5 µl of 10 mg/ml herring sperm DNA as a carrier (CLONTECH #K1606-A), 0.5 µg of pODB80-PDZ IK or pODB80-PDZ LM, and 50 µl of sterilized water were sequentially added thereto. The resulting mixture was stirred well for 1 minute with a vortex and then the plasmid was introduced into the cells by heat shock at 42°C for 20 minutes. After incubation at 42°C, yeast cells were precipitated by centrifugation at 14000 rpm. The cells were suspended in 100 µl of sterilized water and then plated on an SD-Trp agar plate. The SD-Trp plate was prepared with –Trp DO supplement (Clontech #8604-1) according to the manual. The colonies, formed 3 days after incubation at 30°C, were tested for the presence of the introduced plasmid by colony PCR using the above-mentioned KOD dash DNA polymerase and primers 5BD (5'-TCATCGGAAG AGAGTAGTAA C-3') and 3BD (5'-CGTTTTAAAA CCTAAGAGTC AC-3'). The resulting colonies designated PJ69-4A/pODB80-PDZ IK and PJ69-4A/pODB80-PDZ LM were found to contain the respective plasmids of interest and were used for the yeast two-hybrid screening.

Yeast two-hybrid screening

PJ69-4A/pODB80-PDZ IK or PJ69-4A/pODB80-PDZ LM was inoculated in 100 ml of SD-Trp liquid culture medium, and then cultured at 30°C overnight while being shaken at 270 rpm. After being precipitated by centrifugation at 2400 rpm for 5 minutes, the yeast cells were suspended in 300 ml of YPDA culture medium and further cultured at 30°C for 5 hours. The yeast cells were again precipitated by centrifugation at 2400 rpm for 5 minutes, and then suspended in 150 ml of sterilized water. After precipitation, the yeast cells were washed. The following reagents were added successively to the precipitated yeast cells: 14.4 ml of 50% polyethylene glycol 4000, 3.24 ml of 1 M

lithium acetate (pH 8.7), 450 µl of 10 mg/ml herring sperm DNA as a carrier (CLONTECH #K1606-A), 4.89ml of sterilized water, 60 µl of 0.5 µg/µl Human Aorta MATCHMAKER™ cDNA library (CLONTECH #HL4040AH) or Human Prostate MATCHMAKER™ cDNA library (HL4037AH). The cells were suspended by vortexing for 1 minute. They were cultured at 30°C for 35 minutes, then at 42°C for 30 minutes while the mixture was stirred by inverting the tube several times every 5 minutes. The yeast cells were precipitated by centrifugation and the supernatant discarded. The precipitated cells were suspended in 7.5 ml of sterilized water. 250-µl aliquots of the suspension were plated on 150-mm dishes with SD/-Trp/-Leu/-His/+1mM 3-AT. The dishes were incubated at 30°C.

SD/-Trp/-Leu/-His/+1mM 3-AT was combined with 4.0 g of Difco Yeast Nitrogen Base (without amino acids), 0.40 g of Synthetic Complete Drop Out Mix (defined below), 600 ml of distilled water and 10.0 g of Difco Bacto-Agar. The pH of the mixture was adjusted to 5.6 by 10N sodium hydroxide. 12.0 g of glucose was added to the mixture. The medium was autoclaved. After the medium was cooled down to 55°C, 3-amino-1,2,4-triazole (3-AT, Sigma #A-8056) was added thereto at a final concentration of 1 mM, and the medium was poured into plates. Synthetic Complete Drop Out Mix is a mixture that contains 2.0 g of adenine sulfate, 2.0 g of arginine hydrochloride, 2.0 g of isoleucine, 2.0 g of lysine hydrochloride, 2.0 g of methionine, 3.0 g of phenylalanine, 2.0g of serine, 2.0 g of threonine, 2.0 g of tyrosine, 1.2 g of uracil and 9.0 g of valine.

The colonies obtained were transferred onto SD/-Trp/-Leu/-His/-Ade agar plates. The colonies selected on the plates were tested for â-galactosidase by the colony-lift filter assay according to the method as described in the user manual of CLONTECH MATCHMAKER™ "Protocol (PT1030-1) of CLONTECH MATCHMAKER™ Two-Hybrid System2". The selection gave adenine- and LacZ-double-positive colonies. By colony PCR, DNAs were obtained from the selected clones, and then the nucleotide sequences were directly determined from the DNA. The colony PCR was performed by suspending yeast cells from the colonies in 30 µl of a reaction solution (3 µl of 10 × KOD Dash buffer #1 attached to TOYOBO KOD Dash DNA polymerase (TOYOBO #LDP-101),

12 pmoles of primers 5BD (5'-TCATCGGAAG AGAGTAGTAA C-3') and 3BD (5'-CGTTTTAAAA CCTAAGAGTC AC-3'), 0.2 mM dNTP, 1 unit of KOD Dash DNA polymerase). After incubation at 95°C for 5 minutes, PCR amplification was achieved with 40 cycles consisting of denaturation at 98°C for 10 seconds, annealing at 55°C for 2 seconds and extension at 74°C for 30 seconds. The thermal cycling was carried out in a GeneAmp PCR2400 from Perkin Elmer.

PCR products were purified with a QIAquick™ PCR purification kit (QIAGEN #28106) according to the manual. The direct nucleotide sequence determination was performed with primers 5BD and 3BD by using a BigDye™ Terminator Cycle Sequencing FS Ready Reaction Kit (ABI #4303151) according to the manual.

Recovery of plasmid DNA from yeast

Zymoprep™ (Yeast Plasmid Mini-preparation Kit; Funakoshi #D2001) was used for recovering plasmid DNA from yeast cells in which the above-mentioned MATCHMAKER™ Human Brain cDNA had been introduced. By the method according to the manual, plasmid DNA was recovered directly from yeast colonies grown on agar plates by lysing the cells. Each DNA sample contained the plasmid pODB80-PDZ IK or pODB80-PDZ LM DNA and pACT2-derived DNA containing a cDNA insert of MATCHMAKER™ Aorta cDNA or MATCHMAKER™ Prostate cDNA. *E. coli* HB101 was transformed with each of the recovered DNAs, and then the DNAs were prepared on a large scale. The plasmid consisting of pACT2 vector and human brain cDNA insert contains the Leu2 gene as the marker for yeast auxotrophic selection. Because *E. coli* HB101 has a mutant LeuB gene, *E. coli* containing a plasmid derived from pACT2 vector is selectable on a leucine-deprived agar culture medium. The selection was carried out as described in detail in "Yeast Protocols Handbook (PT3024-1)" from CLONTECH.

The cDNAs selected by this method were sequenced. The table below lists proteins with known sequences identified by this method.

OBTP. Galectin-3, Muc12 and BAI3 that bind to the PDZ I-K domain or PDZ L-M comprised in the proteins of the instant invention are proteins having a hydrophobic amino acid region at the C-terminus. Furthermore, it is known that OBTP (Genbank:NP-037529) is highly expressed in breast cancers and Galectin-3 is highly expressed in pancreatitis, pancreatic cancers, and astrocytic tumors (Galectin-1 and galectin-3 in chronic pancreatitis, Lab Invest. 2000 80, 1233-1241; Comparative analysis of galectins in primary tumors and tumor metastasis in human pancreatic cancer, J Histochem Cytochem 2001 49, 539-549; Galectin-3 and galectin-3-binding site expression in human adult astrocytic tumors and related angiogenesis, Neuropathol Appl Neurobiol 1999 25, 319-330). Therefore, these proteins are highly useful proteins that can be used as markers for the above-mentioned diseases. They can be useful also as therapeutic targets for drugs.

On the other hand, Muc12 was isolated as a gene that is down-regulated in colorectal cancer, and BAI3 has been suggested to play a role in suppressing glioblastoma. Therefore, these tumor-suppressive genes are postulated to exert an anti-tumor effect if used in gene therapy.

This indicates that the protein of the present invention is useful for identifying medically important proteins expressed in biological samples.

Table

The column "Clone ID" contains names of clones obtained by yeast two-hybrid (Y2H) system using LM or IK as the bait. The column "matched sequence" contains names of proteins found by homology search to be identical to the polypeptide encoded by the clone. The column "C-terminal" contains the C-terminal sequence from each of the clones.

Clone ID	Bait	matched sequence	C-terminal
4_1_14	LM	galectin-3	YTMI
4_1_25	LM	BAI3	QTEV
3_2_134	IK	OBTP(overexpressed breast tumor protein)	QPGV
IK-A63	IK	Muc12	ASTV

3. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Respectfully submitted,

Date: 2 Oct 2002

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